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STUDY OF NUCLEOIDS IN BEAN CHLOROPLASTS BY FLUORESCENT AND ELECTRON MICROSCOPY

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The DNA containing areas of chloroplasts, so-called nucleoids, were studied within bean chloroplasts by fluorescent and electron microscopy. The use of the DNA specific fluorochrome DAPI (4'6-diamidino-2-phenylindole) makes it possible to examine, *in situ* with fluorescent microscope, some characteristics of organization of nucleoids in chloroplasts. Fully developed bean chloroplasts contain a moderate number (approximately 25—40) of small (dimeter 0.2—0.4 μm), spherical nucleoids randomly distributed throughout the chloroplast. According to these characteristics the bean belongs to the SN (scattered nucleoids) group. Chloroplast nucleoids were also observed by electron microscopy with the use of specific preparative technique for better visualization of nucleoids. These investigations partly confirm the results about the organization of nucleoids obtained by fluorescent microscopy. The nucleoids in dividing bean chloroplasts are organized in the same way as in non-dividing chloroplasts. A similar quantitative portion of nucleoids was determined in dividing (17.4%) and in non-dividing (16.9%) bean chloroplasts by morphometric measurements. It has been established that parallelly with the enlargement of chloroplast size before its division their nucleoids increase as well. They are evenly distributed within each half of chloroplast before the end of chloroplastokinesis. Complete division of chloroplast restores the initial number and distribution of nucleoids in two daughter chloroplasts.

Introduction

Chloroplasts possess their own DNA that is quite different from nuclear DNA but it also has the ability to carry genetic information (Hoober 1984). The areas within the chloroplast stroma that contain DNA are similar to the bacterial nucleus because they are not bounded by membranes. The terms such as chloroplast nuclei, nucleoplasm, plastom and genophore have been used very often to denote the genome of chloroplast. In this paper we prefer the term nucleoids, which also designate the DNA containing areas within the chloroplasts.

The studies of chloroplast nucleoids by light microscopy as well as by electron microscopy have been quite difficult and rather obscure. The unequivocal presence of chloroplast DNA has been demonstrated rarely by Feulgen staining, and rather faint images have been obtained by using acridine dyes and fluorescent microscopy (Bisalputra and Bisalputra 1969, Woodcock and Bogorad 1970). Several investigators applied electron microscopy of serial sections to obtain more information about chloroplast nucleoids (Ris and Plaut 1962, Woodcock and Fernandez-Moran 1968, Herrmann and Kowallik 1970, Odintsova et al. 1970, Possingham et al. 1983). Until now, one of the most promising methods for visualization of nucleoids within the chloroplast is DAPI-fluorostaining technique (Kuroiwa and Suzuki 1980, Selldén and Leech 1981, Possingham et al. 1983, Hashimoto 1985, Miyamura et al. 1986).

The specific fluorescent probe for DNA detection, DAPI (4'6-diamidino-2-phenylindole) synthesized by Dann et al. (1971 cit. James and Jope 1978) is highly specific in its binding to linear polimers with phosphate backbones such as polyphosphates and DNA molecules (Coleman 1979). It was introduced by Williamson and Fennel (1975 cit. Selldén and Leech 1981) who used it to study mitochondrial DNA in yeast cells. It has also been successfully used as a means of detecting mycoplasmas in tissue culture cells (Russell et al. 1975) and to study kinetoplast DNA in trypanosomes (Hajduk 1976). More recent data show that DAPI can be used for visualization and localization of nucleoids within the plastids of various plants (James and Jope 1978, Coleman 1978. Kuroiwa et al. 1981) and suggest that the stain would be an excellent probe for investigations of the organization, character and relative amount of DNA per plastid.

The purpose of the present report was to demonstrate the way in which fluorochrome DAPI can be used to investigate nucleoids in chloroplasts of various plant species. In this study an attempt was made to determine in detail the characteristics of organization of nucleoids in bean chloroplasts by DAPI-technique. In addition to that, changes in chloroplast nucleoids during the chloroplast division were followed. The percentage of nucleoids within the dividing and non-dividing bean chloroplasts was also established by morphometric measurements.

Material and Methods

Bean seedlings (*Phaseolus vulgaris* L. cv. Starozagorski) were soaked in running tap water at 20°C for 24 hours and germinated in a growth cabinet on moist filter paper in the dark at 25°C for 24 hours. The sprouts were transferred to moist vermiculite and grown at 20° C under white

light (two fluorescent tubes, 20 W, 4500 K, illumination intensity 5000 lx at ground level) in a photoperiod of 16 hours light and 8 hours darkness. Young primary been leaves (4 days after sowing) and fully expanded leaves (2 weeks old) were used for investigations. The leaves were cut into small pieces and differently fixed.

For fluorescent microscopy leaf pieces were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH=7.2) for 30 minutes. After fixation the material was washed in cacodylate buffer for 10 minutes and stained with 20 μg/ml DAPI (4'6-diamidino-2-phenylindole) in distilled water for 5—10 minutes. The material was rinsed with cacodylate buffer, transferred onto a glass slide and covered with a glass slip. Before fluorescent microscopic observation, the cover glass was gently pressed against the sample. All investigations were made with an Opton ICM 405 epifluorescent microscope equipped with phase contrast optics. DAPI-DNA complex was excited by a HBO 200 W Hg lamp. Observations of DAPI fluorescence were made with an UV-100 objective and excitation filter (G-365, FT-395) in combination with suppression filter (LP-420). Photomicrographs were taken with Kodak 35 mm Tri-X Pan film using exposure time of 10—30 seconds. To test intactness of chloroplasts, the plastids were examined at any time under phase contrast microscope the criterion being that the phase contrast image of the intact chloroplast was bright and surrounded by a halo.

For electron microscopy leaf pieces were fixed in 1% glutaraldehyde in 0.025 M phosphate buffer (pH=6.8) for 1.5 hour and then in 3% glutaraldehyde in the same buffer for 3 hours at room temperature. After fixation the material was washed with several changes of phosphate buffer on ice for 2 hours and then it was postfixed in 1% osmium tetroxide in 0.05 M phosphate buffer (pH=6.8) at 0° C overnight. The material was dehydrated through a graded ethanol series and embedded in Araldite. The ultrathin sections were stained with uranyl acetate and Reynolds lead citrate for 7 minutes each (Lindbeck et al. 1987). The sections were examined in an Opton 10 A electron microscope.

Morphometric measurements were performed on photomicrographs of 30.000 times final magnification on which a 5 mm square lattice was aditionally copied (Modrušan and Wrischer 1987). The quantitative portion of nucleoids in the dividing and non-dividing chloroplasts was established from the number of crossings of the lattice (hits) that cover a surface section of nucleoids and chloroplasts. The percentage of nucleoids inside the chloroplast was calculated as a mean value of 30 measurements and the results obtained per surface section of chloroplast were expressed per chloroplast volume. Standard error was reckoned according to Campbell (1971).

Results and Discussion

The value of DAPI as a stain for the visualization of chloroplast nucleoids lies in its very high quantum efficiency of fluorescence which is enhanced more than tenfold by binding to DNA (Lin et al. 1977 cit. Coleman 1979). DAPI promotes a far more intense fluorescence of DNA than acridine orange used in earlier studies (Ris and Plaut 1962, Woodcock and Bogorad 1970) and thus facilitates investigation of chloroplast nucleoids (James and Jope 1978, Kuroiwa and Suzuki 1980). The method also has an advantage over autoradiographic

methods (Hermann 1970, Gibbs and Poole 1973, Rose et al. 1974, Possingham 1983) since the stain visualizes all the chloroplast DNA, not only the portion which has recently been synthesized. As an alternative to electron microscopy the use of DAPI has the advantage that it avoids the electron microscopic preparative technique that could alter the arrangement of nucleoids within the plastids. The nature of the stained material within the chloroplasts is easily demonstrated by applying DNase, which causes the loss of fluorescence (James and Jope 1978). On the other hand high specificity of DAPI for DNA can be achieved by pretreating the material with RNase and perchloric acid which remove interfering materials (Coleman 1979).

Fluorescent photomicrograps (Figs. 1 and 2) present a cell from bean leaf tissue after DAPI staining. The main fluorescent area is the cell nucleus while small fluorescent dots grouped in oval areas are chloroplast nucleoids. Every portion of the nucleus, except the nucleolus, is fluorescent (Fig. 2). The reason why the nucleolus shows little or no flourescence is the high degree of DAPI specificity for DNA but not for RNA (James and Jope 1978). The intensity of nuclear staining is so high that the fluorescence of chloroplast nucleoids was hardly revealed photographically at the same exposure (Figs. 1 and 2). Figure 3 consists of photomicrographs of the same group of DAPI stained bean chloroplasts using both phase contrast (Fig. 3a) and fluorescent microscopy (Fig. 3b). These sets of photomicrographs show several distinct aspects of the chloroplast structure. The phase contrast image of chloroplasts clearly shows defined grana as well as the division profile of chloroplasts. These facts confirm that the chloroplasts were kept structurally intact during the observations. Therefore, it can be concluded that the random distribution of nucleoids appearing in the fully developed chloroplasts (Fig. 4) was not an artefact, which may occur if plastids lose their intactness. Fluorescent photomicrographs (Figs. 3b. 4 and 5) show a few other facts: firstly, the in-depth distribution of fluorescent elements within the chloroplasts; secondly, the variable amount of fluorescence per plastids; thirdly, the different intensity of fluorescence depending on the orientation of chloroplasts.

A moderate number of nucleoids with indistinct contour was found in fully developed bean chloroplasts (Fig. 4) by moving the focal plane. Individual nucleoids cannot always be resolved and sometimes their aggregate appearance represents several nucleoids adjacent to one another. It is not possible to obtain an accurate count of their number but an estimate of as many as 25 to 40 nucleoids has been made for many bean chloroplasts (Fig. 4). The majority of nucleoids were located in the central region of the chloroplast. From photomicrographs taken at different focal planes, it seems that the nucleoids are randomly scattered throughout the chloroplast (Fig. 4). The nucleoids within bean chloroplasts are small (diameter 0.2—0.4 μm) and they are spherical in shape. According to these characteristics the bean chloroplasts (Fig. 4) belong to the SN (scattered nucleoids) group of the classification based on number, size, shape and distribution of nucleoids (Kuroiwa et al. 1981). The SN group is the most common arrangement of nucleoids in chloroplasts of various plant species and it has been found in chloroplasts of three other dicotyledons: *Brassica, Vicia* and *Pisum* (Kuroiwa et al. 1881). Such characteristics of chloroplast nucleoids as the number, size, shape and distribution are relatively constant in fully developed chloroplasts of each plant species (Kuroiwa et al. 1981). The distribution of nucleoids

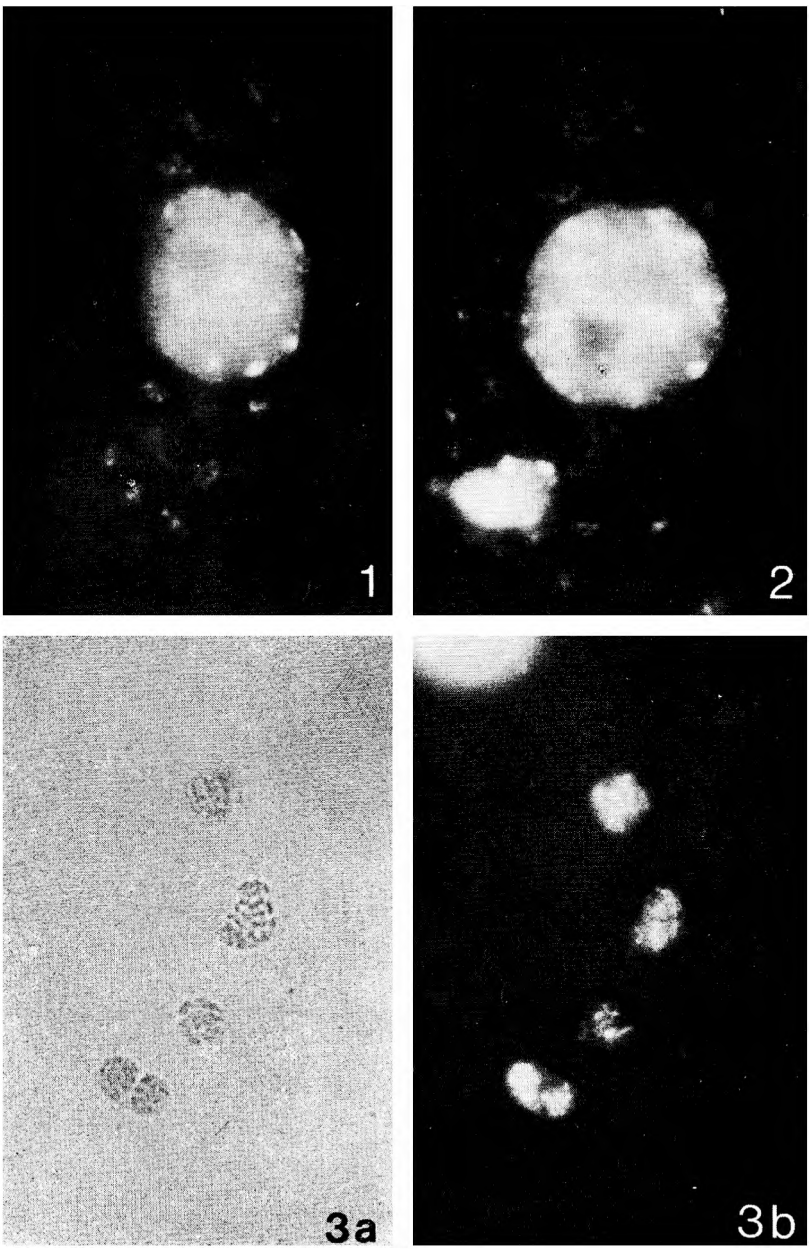

Fig. 1. Fluorescent photomicrograph of DAPI stained cell (nucleus and chloroplasts) from bean leaf tissue. 1,500 : 1.

Fig. 2. Fluorescent photomicrograph of DAPI stained nucleus. The dark spot inside the nucleus marks the position of the nucleolus. 1,500 : 1.

Fig. 3. Phase contrast (3a) and fluorescent (3b) photomicrographs of the same group of bean chloroplasts stained with DAPI. 1,700 : 1.

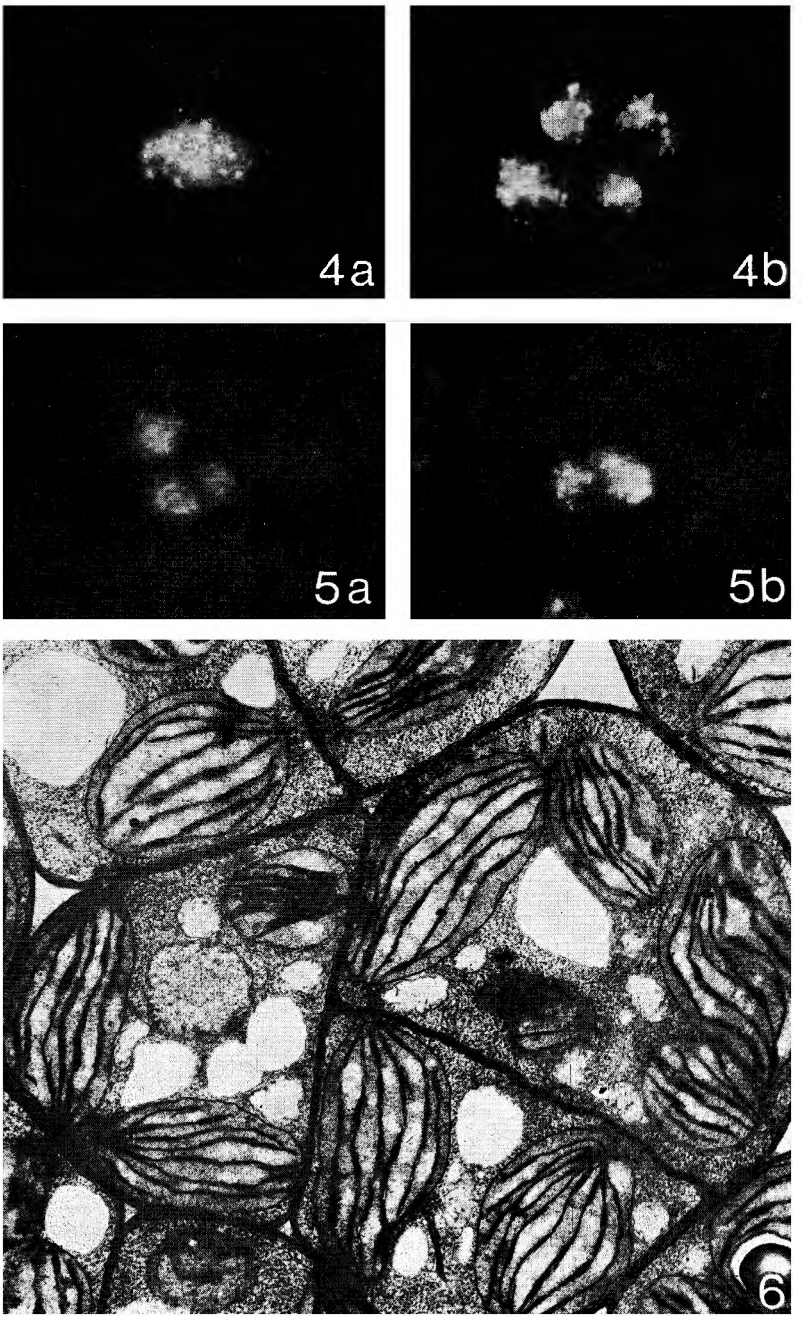

Fig. 4. Fluorescent photomicrographs (4a and 4b) of fully developed bean chloroplasts after DAPI staining. 2,500 : 1.
Fig. 5. Fluorescent photomicrographs (5a and 5b) of DAPI stained dividing bean chloroplasts. 2,000 : 1.
Fig. 6. Electron micrograph of chloroplasts with nucleoids (electron translucent areas) in young bean leaf tissue. 9,000 : 1.

changes in the course of chloroplast differentiation (Selldén and Leech 1981, Possingham et al. 1983, Miyamura et al. 1986, Nakamura et al. 1986) and its division (Rose et al. 1974, Lüttke and Bonotto 1981, Hashimoto 1985). The number of nucleoids also varies in some stages of plastid division. It has been established that the number of nucleoids is in correlation with chloroplast size (Kuroiwa et al. 1981). Our investigations demonstrate that the nucleoids within the dividing bean chloroplasts (Fig. 5) are organized in the same way as in non-dividing chloroplasts. However, the number of nucleoids changes remarkably during the chloroplast division (Fig. 5). The quantitative portion of nucleoids within the stroma of dividing and non-dividing bean chloroplasts was determined by morphometric measurements (Table I). It was established that the percentage of nucleoids in dividing (17.4%) was nearly the same as in non-dividing chloroplasts (16.9%). These results are presented in Table I and it is significant to note the difference

Table 1. The number of hits (mean value with standard error indicated) per section surface of chloroplast or nucleoid and the percentage of nucleoids within the dividing and non-dividing chloroplasts.

	Number of hits per surface section of chloroplasts	Number of hits per surface section of nucleoids	Percentage of nucleoids
DIVIDING CHLOROPLAST	290 (±20.4)	49 (±4.9)	17.4%
NON-DIVIDING CHLOROPLAST	187 (±13.4)	33 (±3.9)	16.9%

in the volume between dividing and non-dividing chloroplasts. It has been established that the chloroplast nucleoids increased parallelly with the enlargement of chloroplast before its division. Nucleoids are semi-synchronously divided and they are distributed evenly within each half of chloroplast before chloroplastokinesis. Complete division of chloroplast restores the initial number and distribution of nucleoids in two daughter plastids (Kuroiwa et al. 1981).

A specific preparative method which enhances the contrast of chloroplast nucleoids (Lindbeck et al. 1987) was used for investigation of nucleoids by electron microscopy. By this method the nucleoids are visualized as electron translucent areas within the bean chloroplasts (Fig. 6). These areas are spread throughout the chloroplast stroma between the existing thylakoids and adjacent to them. This observation is consistent with the results obtained by DAPI staining and fluerescent microscopy. Inside the electron translucent areas, fibrillar structures that represent DNA molecules can sometimes be obviously seen (Hashimoto 1985, Lindbeck et al. 1987). These fibriles can be associated with thylakoid membranes and with the inner membrane of the chloroplast envelope. The morphological association between chloroplast DNA and internal chloroplast membranes as well as a number of putative roles of this association have been presented by several investigators (Rose and Lindbeck 1982, Hansmann et al. 1985, Lindbeck and Rose 1986). One of the most probable roles of thylakoid-bound chloroplast DNA is partitioning of nucleoids at chloroplast division (Rose et al. 1974, Rose and Possingham 1976, Rose 1979).

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SAŽETAK

FLUORESCENCIJSKO-MIKROSKOPSKA I ELEKTRONSKO-MIKROSKOPSKA ISTRAŽIVANJA NUKLEOIDA KLOROPLASTA GRAHA

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Nukleoidi kloroplasta graha, tj. područja u stromi tih kloroplasta koja sadržavaju molekule DNA, istraživani su fluorescencijskim i elektronskim mikroskopom. Primjenom DNA-specifične fluorokromne boje DAPI (4'6-diamidino-2-fenilindol) moguće je *in situ* fluorescencijskim mikroskopom utvrditi neke karakteristike organizacije nukleoida unutar kloroplasta. Tako je utvrđeno da kloroplasti graha sadržavaju srednji broj (otprilike 25—40), malih (promjer 0.2—0.4 μm), sferičnih, rastresito raspoređenih nukleoida. Prema tim karakteristikama nukleoida kloroplasti graha pripadaju tzv. SN (scattered nucleoids) grupi. Elektronsko-mikroskopskim istraživanjima mladog lisnog tkiva graha uz primjenu preparativne metode za bolje prikazivanje nukleoida potvrđeni su rezultati o distribuciji nukleoida dobiveni fluorescencijsko-mikroskopskim istraživanjima. Tijekom diobe kloroplasta graha ne dolazi do značajnije promjene u organizaciji nukleoida. Morfometrijom je utvrđen približno jednak udio nukleoida kod kloroplasta u diobi (17.4%), kao i kod kloroplasta izvan diobe (16.9%). Ustanovljeno je da se područje koje zapremaju nukleoidi povećava paralelno s porastom veličine kloroplasta prije njegove diobe. Nukleoidi se jednoliko raspodijele unutar svake polovice kloroplasta prije završetka njegove diobe. Diobom kloroplasta uspostavlja se početni broj i distribucija nukleoida unutar dva nova plastida.

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